Original Research Article

Antimicrobial Resistance Profile of Airborne *Aspergillus flavus* and Methicillin-Resistant *Staphylococcusaureus* in Public Toilets within Port Harcourt.

ABSTRACT

Aspergillus flavus and Methicillin-resistant *Staphylococcus aureus* (MRSA) resistance to both antibiotics and antifungal medications put the public at risk.This study therefore was carried out to investigate the antimicrobial susceptibility pattern of *Aspergillus flavus* and Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from publicly used toilets in Port Harcourt, Rivers State. Eighty (~~80~~) samples were collected for a period of two months from five public toilets using the sedimentation air sampling method. Samples were subjected to identification, antibiotics and antifungal susceptibility test using Kirby-Bauer disk diffusion method, plant extracts and molecular identification of bacterial isolates for analysis. The susceptibility profile showed that *MRSA* were resistant to Ofloxacin (61%), Ceftazidime (92.30%), Levoflaxcin (92.30%), Vancomycin (77 %),Gentamycin (61%), Azithromycin (46.2%) and cefotaxime (46.2%) and susceptible to Imipenem (100%), Meropenem (92.32%). Ketoconazole and nystatin both antifungals were both effective on the *A. flavus*. Methanol extract of *Ocimumgrastissimum* was more effective followed by *Psidium guajava* and *moringa oleifera* on MRSA but also less effective in *A. flavus*. The MAR index ranged from 0.1 to 0.8 which showed that 60% of *MRSA* isolates had MAR index of 0.8, while 20% had MAR index of 0.4 and 0.5. The antimicrobial activity of the extracts is promising as the extracts could be used as a cheap antimicrobial for the treatment of infections cause by these test organisms. MRSA is a major contributor to skin infections, bloodstream infections, toxic shock syndrome, and joint inflammation. *Aspergillus flavus* can result in a range of health issues these problems include allergic reactions, aspergilloma, both invasive and non-invasive. infections are a matter of importance for public health. Conclusively, this study revealed both organisms present in toilet air, their vulnerability patterns was established, their resistance gene verified, and explore the potential use of natural plant compounds on them, would assist in mitigating public health.

Comment [BI1]: Mention the country

Comment [BI2]: (*n*=80)

Comment [BI3]: Isolation and identification

Comment [BI4]: Mention the % of isolation of staph and Aspergillus

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Keywords: MRSA, *Aspergillus flavus*, Susceptibility pattern, Plant extract, Public health, Multi-drug resistance, Resistance

Introduction

Aspergillus Flavus

Aspergilli has continuously existed in the human environment. Micheli was the first person to identify the separate parts of fungi, such as the stalks and spore heads. However, it took until the mid-19th century for people to realize that these fungi were actually responsible for

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causing decay, diseases in humans and animals, and for producing useful metabolic products through fermentation. (Raper & Fennel, 1965). *Aspergillus flavus*, originally identified by Link in 1809, now refers to not only a single species but also a cluster of closely associated species. *A. flavus* is the second in rank, following only *A.fumigatus* is responsible for human invasive aspergillosis. Furthermore, it is the primary *Aspergillus* species that invades insects (Campbell, 1994). Additionally, it has the ability to induce illnesses in economically significant crops like maize and peanuts, as well as generate highly toxic substances called mycotoxins.

*A. flavus*fungus is responsible for a wide range of illnesses in people, ranging from allergic reactions to invasive infections that affect blood vessels. After *A. fumigatus*, *A. flavus* can be the second most common reason for both invasive and non-invasive aspergillosis(Denning, 2003; Morgan *et al.*, 2005).The main way the infection spreads is when someone breathes in the spores of the fungus. The larger dimensions of *A. flavus* have a larger diameter of 25 mm, while *A. flavus* has a smaller diameter of 23 mm. *A. fumigatus* has a tendency to be deposited in the upper respiratory tract. Perhaps this could be one explanation for *A. flavus* is frequently responsible for causing fungal sinusitis and skin infections, but it does not typically lead to invasive fungal pneumonia. It is possible that other traits of the spores, in addition to their size, play a crucial role in determining their localization (Morrow *et al.*, 1980).

Methicillin-Resistance Staphylococcus ~~Aureus~~

MRSA, also known as Methicillin-resistant *Staphylococcus aureus*, is a highly thriving and effective pathogen in contemporary times. The organism, which can live peacefully with another organism and is passed on in both healthcare and community environments, is also a primary source of bacteraemia, endocarditis, infections of the skin and soft tissues, infections of bones and joints, and infections acquired in hospitals. The epidemiology of MRSA is mainly defined by the recurring appearance of epidemic strains due to their genetic diversity.

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Even though the occurrence of MRSA has decreased in certain areas, it still presents a serious risk in the medical field, leading to consistently high rates of illness and death. Successfully treating a medical condition continues to be difficult and necessitates assessing new ways to combat infections as well as supplementary components of care, including seeking guidance from infectious disease specialists, conducting echocardiography, and implementing measures to control the source of the infection. In situations where the protection of the skin is compromised or the immune system is weakened, *Staphylococcus aureus* changes its ~~behavior~~from being a harmless resident to becoming the primary cause of skin infections. **(**Zavadinack*et al*., 2001).This resistance is caused by beta-lactamases known as penicillinases, which are enzymes that break down the beta-lactam ring of the antibiotics found in the beta-lactam group, including penicillin, rendering them ineffective. (Tavares, 2000. Que, Moreillon*et al*., 2009).~~The initial strain of *S. aureus* bacteria that was resistant to methicillin.~~ The first recorded instance of the Methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial strain in England occurred in 1961 when it was discovered in a patient who was receiving medical care in a hospital. Shortly after, other European countries, as well as Japan, Australia, and the United States, began reporting similar cases, leading to the spread of this microorganism and making it the primary cause of infections acquired in hospitals. (Boyce, *et al*., 2005). MRSA, ~~also known as *S. aureus* strains,~~have developed resistance to all beta-lactams, except for cephalosporins that have anti-MRSA properties, as well as combinations of beta-lactamase inhibitors. (Mimica *et al*., 2007).

The understanding of MRSA spread between animals and humans has been significantly influenced by the One Health approach. (Casey, *et al*. 2014). ST398 (CC398) has been extensively documented as a source of livestock-related community-acquired methicillin-resistant *Staphylococcus aureus* in Europe starting from 2005 (Witte *et al.*, 2007). ST398 has been identified as a source of livestock-connected MRSA in Asia, Australia, and the Americas. However, it is important to note that there are other strains present in livestock as

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well.(Smith *et al.*, 2009). Interspecies transmission of MRSA may create added evolutionary limitations, as certain genetic markers associated with evading the immune system, like scn, chp, and sak, seem to show divergent selection. These genetic markers are positively linked to human infection but negatively linked to colonization in livestock. (Sung, & Lindsay 2008).

Comment [BI22]: gene names should be italic

MATERIALS AND METHODS

Study Area

The study area was Port Harcourt Metropolis, Rivers State, Nigeria. Five major locations were chosen from the study area; Mile 3 Market, Nkpolu-Oroworukw Port Harcourt (4.8042° N, 6.9924° E) Rumuokoro, Motor Park (4.8670° N, 6.9944° E), Mile 1 Market Rumuwoji (4.7918° N, 6.9986° E) Model Girls Secondary, School Rumueme (4.83835,6.99588° E) and Rivers State University Nkpolu-Oroworukw (4.8522622, 6.9896428° E). These five (5) locations were selected because the high rate of human activities within the locations leading to spread of infections.

Toilet Air Sample Collection

Comment [BI23]: ref of the methods

The method of sampling used in this investigation was the direct sedimentation method of aerosol sampling which involves the aseptic exposure of growth media including Sabouraud' dextrose agar and Mannitol salt agar both produced by (Titan Biotech Limited, Bhiwadi, india) to the environment air. The agar plates used for the investigation were prepared in duplicates in the laboratory and transported to the point of sample collection aseptically. The agar plates containing adequate amounts of SDA was supplementedwith Ampicillin and tetracycline and MSA were exposed in each of the toilet at various sampling stations and labelled adequately. Exposure period for MSA plates was for 10 Minutes and SDA plates for 1hour and users of the toilets were prevented for a period of 4 hours to prevent inaccurate sample collection as a result of flushing and agitating the toilet water thereby emitting more aerosols.

Microbiological Analysis

Preparation of Plant Sample for Extraction

This approachwas carried out with accordance to Ogbonna *et al.*2010, the plants were left to dry for a period of five days at normal room temperature before being transformed into a powdered state by crushing them. Afterward, 200g of the powder was put into their respective 500ml Erlenmeyer flasks. These flasks already had 200ml of both methanol and sterile water. For a duration of three days, every sample was passed through Whatman No 1 filter paper into a 500ml vessel. Subsequently, the container was subjected to drying at a temperature of ~~400C~~ using an oven drier. After the extracts were dried, 20ml of a solution containing DMSO was mixed with the extracts and placed in the refrigerator for future use

Characterisation and Identification of bacterial and Fungi Isolates

Discrete colonies were picked based on their cultural, morphology, macroscopic and microscopic examinations and biochemical tests. The isolate were subculture on solid NA and SDA and subsequently on slants of the respective agar media and preserved at refrigeration temperature. Identification of the isolates as bacteria and fungi was carried out as described in

(Cheesbrough*et al.*, 2006)

SusceptibilityAssay of the Bacteria isolates

Antimicrobial sensitivity test was performed using Kirby-Bauer method to measure the ability of an antibiotic to inhibit bacteria growth in vitro by disc diffusion (CLSI, 2020). The pure cultures of bacteria isolated were aseptically inoculated into 5ml of sterile peptone water and incubated at 37^0C for 18-24hours. A turbid suspension of the isolate was made in distilled water using 0.5 McFarland Standard prepared as a comparator. A sterile swab was dipped into the bacteria suspension, pressed on the side of the test tubes to allow excess drip off and then evenly smeared on the entire surface of the Mueller Hinton agar. Sterile forceps was used to position the commercial single antibiotic discs (Mast Group Ltd. Mast House

Comment [BI24]: State the name of the plant you used and the character of the extract you will prepare
Also, you need to explain the mode of action of the extract on both bacteria and fungi

Comment [BI25]: pls correct the temp (400 °C or 40 °C) I am really confused

Comment [BI26]: please clearly explain this step to answer the following questions:
1- did you identify all the grown colonies
2- did you select only colonies that match the criteria of S. aureus
3- if the answer of the Q2 is yes, what methods you used for confirming S. aureus isolated

Comment [BI27]: In a separate table mention each antibiotic used and most importantly the concentration of the antibiotic per each disck

Bootle, Merseyside, U.K) on the medium containing each of the test bacteria and incubated at 37^0C for 24hours and zone of inhibition were measured and interpreted as susceptible, intermediate or resistance in accordance with (CLSI, 2020). Multiple antibiotic resistance index MARI were determined. MARL= Number of antimicrobial an organism was resistant to / Total number of antimicrobial the organism was subjected to.

Comment [BI28]: Then you mean that all isolated bacteria not only staph???!!!!

Comment [BI29]: Mention the ligand of each measurement

Comment [BI30]: Mention each antifungal you used with its concentration

Results

A total 16 isolates were obtained from the locationswith 8 isolates identified as methicillin susceptible *Staphylococcus aureus* (MSSA), 5 isolates as methicillin resistant *Staphylococcus aureus*(MRSA) after the use to Methicillin makers which are Oxacillin and Cefoxitin,while 3 isolates were identified as *Aspergillusflavus*.

Comment [BI31]: You mean that 16 staph isolates ???!!!
What about other colonies
Did all 80 samples gave only staph ?
Please clarify this issue as follow:
1- number of samples
2- number of each isolate
3- there distribution of each isolate among the 5 places you include in your study

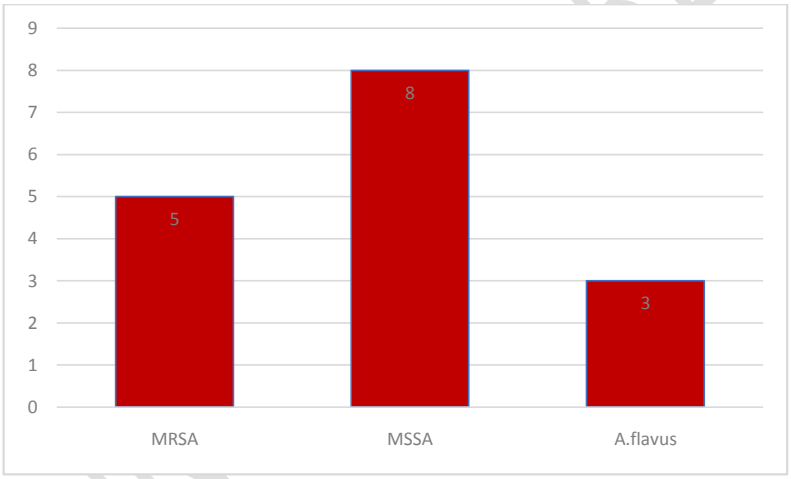

Fig 1:Susceptibility pattern from different Locations

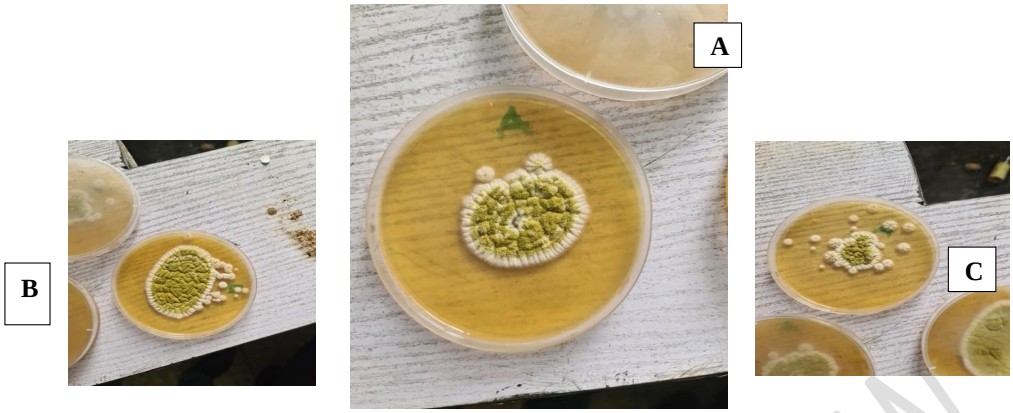

Plate 1: **Identified *Aspergillus Flavus***

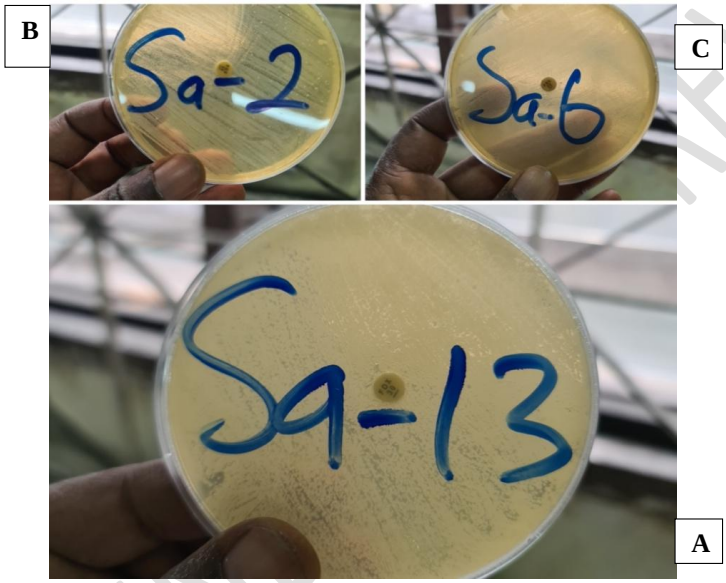

Plate 2:Confirmed *MRSA* after Using the Second Maker (Cefoxitin)

Comment [BI32]: You should include a photo for the susceptible isolates

Antibacterial susceptibility pattern of the bacterial and MAR index are presented in Table's 1 and 2. The Analysis of the *S.aureus* susceptibility pattern, the majority of *S.aureus* isolates were sensitive to Imipenem (100%), followed by Meropenem (90.32%), Oxacillin (53.8%) and Cefoxitin (38.5%). Ofloxacin (100%), Ceftazidime (92.30%), Levoflaxacin (92.30%), Vancomycin (77%), Gentamycin (61.5%), Azithromycin (46.2%), Cefotaxime (46.2%) and were the drugs with the resistance. Therefore, in this study, it was found that 60.0% of the

MRSA isolates had a MAR score above 0.8, as indicated by the Multiple Antibiotic resistance (MAR) index. 60% of the MRSA isolates examined in this study had a MAR index higher than 0.2, as indicated by the Multiple Antibiotic Resistance index. It is important to note that areas where antibiotics are frequently used and sources of contamination typically have MAR index values greater than 0.2 (Davis *et al*.**,** 2004; Krumperman, 1985).

Table1: Susceptibility Profile of *MRSA* to 12 Antibiotics

Antibiotic Class/Conc. (µg)	**Resistant n (%)**	**Intermediate n (%)**	**Susceptible n (%)**
Cephalosporin			
Cefuroxime (30) CXM	8(61.5)	5(38.5)	0(0.00)
Cefotaxime (30) CTX	6(46.2)	7(53.8)	0(0.00)
Ceftazidime (10) CAZ	12(92.30)	0(0.00)	1(7.7)
Aminoglycoside			
Gentamicin (10) GEN	8(61.5)	0(0.00)	5(38.5)
Macrolides			
Azithromycin. (10) AZM	6(46.2)	2(15.4)	5(38.4)
Carbopenem			
Imipenem (10) IMI Meropenem (10) MEM	0(0.00)	0(0.00)	13(100)
Fluoroquinolones			
Ofloxacin (5) OX	13(100)	0(0.00)	0(0.00)
Levofloxacin (5) LEV	12(92.30)	0(0.00)	1(7.7)
Glycopeptide			
Vancomycin (30) VAN	10(77)	0(0.00)	3(23)
Cephamycin			
Cefoxitin (30) OX	5(38.5)	0(0.00)	8(61.5)
Penicillin			
Oxacillin (10) FOX	6(46.2)	0(0.00)	7(53.8)

Comment [BI33]: What is the threshold or level by which you determine each category

Table 2: MAR Indices of Methicillin-Resistance S*taphylococcus aureus* (MRSA) Isolated during the Study

MAR Index	*MRSA* N=5
0.1	0(0.00)
0.2	0(0.00)
0.3	0(0.00)
0.4	1(20.0)
0.5	1(20.0)
0.6	0(0.00)
0.7	0(0.00)
0.8	3(60.0)

Key: Multiple Antibiotic Resistance (MAR)

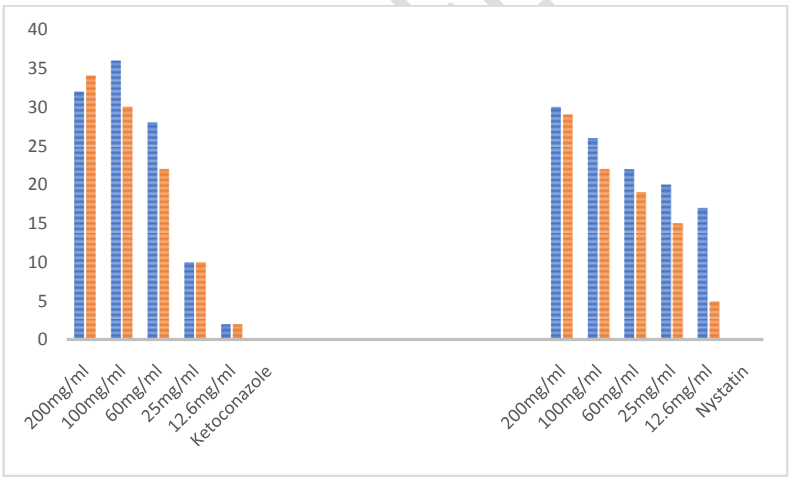

Fig 2: Susceptibility pattern of Antifungal drugs

Discussion

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where is the corresponding graph for MRSA

Comment [BI36]: where is the data of Plant Extract

The findings of the antibiotic sensitivity patterns in this study revealed that MRSA bacteria were sensitive to Imipenem (100%), Meropenem (90.32%), and Oxacillin (53.8%). Which corroborates previous report of other investigation sensitivity of these drugs to MRSA (Fan *et al.* (1986) and Kayser *et al*. (1989).The susceptibility pattern of MRSA showing resistance with Vancomycin at resistance rate of 77% due to its ability to hampers the peptidoglycan layer of bacterial cell walls but also hinders bacteria from effectively carrying out cell growth and division which aligns with Schultsz*et al*. in 2012.Gentamicin had a resistance rate of 61.5% with its ability to a permanent binding to the 30S ribosomal subunits, disrupting the creation of messenger RNA.(R.R Culter *et al*., 1983). The organisms in this study have a strong resistance to the beta-lactam antibiotics (Cefuroxime 61.50%, Cefotaxime46.2%and Ceftazidime92.30%.) due to the widespread and unregulated use of these antibiotics, their low cost, and the acquisition of the blaCTX, blaSHV, and blaTEM genes. Nevertheless, the resistance to beta-lactam medications aligns with the findings of Bedasa*etal*., (2018). Ofloxacin had resistance rate of 100% and Levofloxacin with a resistance rate of 92.30%. were generally in agreement with report by other investigators (Christof *et al*., 1996)

Azithromycin at 46.2% with its ability to reduce the production of α-hemolysin and biofilm formation in *Staphylococcus aureus*accordance with Gui *et al*., 2014.

The levels of the *Psidium guajava* extracts that were examined against *Staphylococcus aureus* resulted in different sizes of zones of inhibition around the discs soaked in the extracts. These ranges varied from 2.00 mm to 18.00 mm, showing that all the bacteria were responsive to at least one of the extracts. The variation in inhibition halo sizes ranged from 4mm to 17mm. The methanol extract was more effective at stopping bacterial growth compared to the aqueous solvents. The extracts and essential oil from the *Psidium guajava* plant are highly potent against MRSA, suggesting they could be valuable sources of new antimicrobial substances. This aligns with the findings reported by Gonçalves *et al* (2008).

Comment [BI37]: this is the mode of action of the drug, not the cause or resistance.

Comment [BI38]: Please remove all sentences that describe the mode of the action of the drug and replace it with the cause of resistance (ex. The misuse of antibiotics , plasmids that transfer the resistance , ….)

Comment [BI39]: You did not mention the sizes of the inhibition at all in the results

Comment [BI40]: Where is these results, You did not mention it in the result section

The impact of guava leaf extracts was more significant on MRSA. The findings of the study indicated that *Moringa oleifera* leaf extract obtained with methanol demonstrated a wide range of effectiveness against various bacterial strains. The largest area of inhibition observed at a concentration of 100mg/ml was 21mm, but for the MRSA, the smallest area at a concentration of 12.5mg/ml was only 2.0mm. Comparatively, the largest zone of inhibition at a concentration of 100mg/ml was 15mm for the *A. flavus*, and the smallest zone at a concentration of 12.5mg/ml was 3.0mm. The methanol extract of flavus continues to significantly inhibit further growth. *Moringa oleifera* contains a range of beneficial plant compounds such as alkaloids, flavonoids, glycosides, saponins, and tannins. The current research study provided clear evidence of *Moringa oleifera's* ability to inhibit the growth of *A. flavus*. The study found that the methanol extracts of *Ocimumgratissimum* showed strong inhibitory effects on *Staphylococcus aureus* and *A. flavus* This suggests that *Ocimumgratissimum* has antimicrobial properties. The methanol extract showed greater inhibition in comparison to the aqueous extract. This can be determined by the fact that methanol has the capability to extract a larger amount of crucial oils and secondary compounds from plants, which are thought to have antibacterial properties on the test organisms. These compounds have demonstrated strong effectiveness in inhibiting the growth of both gram positive and gram negative bacteria, as well as fungi. This aligns with the findings of the study conducted by Hamma *et al*., 2020.

Ketaconazole is a type of imidazole medication that hinders the conversion of blastospores into the invasive mycelial form. This hindrance likely assists the function of immune cells and could be the main reason for eliminating the infection. Imidazoles are part of a category of antifungal drugs known as azole antifungals, which also include medications such as ketoconazole, miconazole, and clotrimazole. The triazoles, which consist of fluconazole, itraconazole, and voriconazole, are another group of azoles. Nystatin belongs to a group of

Comment [BI41]: ref

Comment [BI42]: you should include the results with data you obtained from your experiment

Comment [BI43]: include it in the results
Also add photos of the bacteria with inhibition zones for documentation
you need also to visualize these results in graphs or tables

antifungal drugs known as polyenes. Its function is to prevent the development of fungi that lead to infection.

Nystatin exhibited a wider range of inhibitory zone diameters, ranging from 29mm to 5mm, while ketoconazole displayed variability between 35mm and 8mm. Ketoconazole has been found to have a beneficial impact on reducing well-known *A. flavus* means yellow. This study is similar to Oji, *et al*. (1982)research, it was found that Ketoconazole has the ability to prevent and treat keratomycosis in rabbits. The antifungal effect of Nystatin is successful in combating fungus type *A. Flavus* is typically slower than many other types of *Aspergillus*. This aligns with a study conducted by Valerie (1965).

Conclusion

The result showed that *Staphylococcus aureus* (MRSA) was the highest prevalent organism. This report revealed that *Ocimumgratissimum*has more antimicrobial effect against both MRSA and *Aspergillus flavus*, compared to other plant extract.It also showed that MRSA isolates are resistant to Ofloxacin, Ceftazidime, Levoflaxacin, Vancomycin, Gentamicin, Azithromycin, Cefotaxime. The drugs that had no effect on the isolates may have no therapeutic value for infections caused by these isolates. This can pose a serious public health problem as the mentioned bacterial species can cause life-threatening infections.

The MRSA bacteria were vulnerable to Imipenem, Meropenem and had limited susceptibility to Oxacillin. This suggests that these medications could potentially be useful in treatment, as the organisms that were tested have no ability to resist them. As a result, they have the potential to be utilized in the treatment of infections that are caused by the bacterial species previously referenced. Ketoconazole and nystatin both showed influences on the *A.flavus* Ketoconazole is capable of preventing and treating certain conditions.

REFERENCES

Comment [BI44]: update the references (30% of the references should be within the last 5 years)

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